Material & Method

Chapter 4

Material and Method

4.1 Plant collection and authentication

The Bark of *Tecomella undulata*, Seed of *Sesamum indicum*, and fruit of *citrus medica* were obtained from the Dabhoi district's surrounding area: district: Baroda, and the local area of Rajkot in May 2021, respectively. The plants were identified and authentified by Dr. Rutva Dave, Assistant Professor, Department of Botany at H.&H.B. Kotak Institute of Science, Rajkot, Gujarat, India as shown in figure 4.1, 4.2, 4.3. The herbariums (BKMGPC/01-03/2022) were preserved in the Department of Pharmacognosy at B.K. Mody Govt. Pharmacy College, Rajkot, Gujarat, India. Subsequently, the bark was washed with water, fruits were sliced, seeds and were left to air dry. Once dried, it was finely powdered using a mixed grinder (MG Livo MX-151, Maharaja mixer grinder) and sieved through a mesh (420). The resulting fine powder was stored in an airtight container for future use.

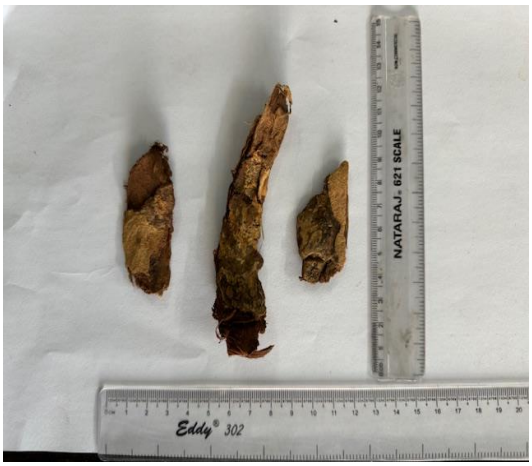

Figure 4.1: Bark of *Tecomella undulata*

Figure 4.2: Fruit of *Citrus medica*

Figure 4.3: Seed of *Sesamum indicum*

4.2 Extraction Method

The powder of all plants separately underwent an exhaustive hydroalcoholic (30:70) extraction using the maceration method at room temperature for 24 hours. The resulting solutions were filtered through the Whatman filter paper (No.42).

Subsequently, the filtrates were recovered using a rotary film evaporator at 40°C, and thoroughly dried. The dried weight of crude extracts was determined and designated as *Tecomella undulata* methanolic extract (TUME), *Citrus medica* methanolic extract (CMME), and *Sesamum indicum* methanolic extract (SIME), respectively, and stored in a sealed jar at 4°C for future use. These crude extracts were referred to by the same abbreviations throughout the text.

4.3 Preliminary phytochemical screening of crude extracts

All three crude extracts were subjected to qualitative phytochemical screening for the presence of various secondary metabolites (Khandelwal KR 2009).

4.3.1 Materials

Test tubes, soxhlet apparatus, desiccators, digital electronic weighing instruments, distillation apparatus, chromatographic paper, and TLC plates were used. All the chemicals are of Analytical Grade Reagent of Angel Tadelinks, Rajkot, Gujarat. The HPTLC system consists of a TLC scanner III (Camag), a spot application device (Linomat 5), a twin trough plate development chamber, and software (Wins Cats).

4.3.2 Preliminary phytochemical screening (Kokate, 2001; Khandelwal, 2001)

Detection of Alkaloids:

Separately dissolve all extracts in dilute hydrochloric acid and filter. The filtrates were evaluated with different alkaloid reagents.

Mayer's test:

To the filtrates, Mayer's reagent (potassium mercuric iodide) was added. The detection of a yellowish cream precipitate showed the presence of alkaloids.

Wagner's test:

Add Wagner's reagent (iodine in potassium iodide) to the filtrates. The formation of reddish-brown precipitate showed the presence of alkaloids.

Dragendorff's test:

To 2–3 milliliters of the filtrates, add Dragendorff's reagent (potassium bismuth iodide solution). An orange-brown precipitate's development indicated the presence of alkaloids.

Hager's test:

To two to three milliliters of the filtrates, add Hager's reagent (saturated picric acid solution). The presence of alkaloids was shown by the production of a yellow-colored precipitate.

Detection of Glycosides:

Glycoside assays were performed on the hydrolysate obtained from the hydrolysis of extracts using diluted HCl.

Modified Borntrager's test:

Add dil. HCl and FeCl3 solution to the extracts. Put the pot on to boil for approximately five minutes. After cooling, shake with the same volume of organic solvent (benzene, for example). After being divided, the benzene layer was treated with an equivalent volume of ammonia solution. The presence of anthracene glycoside was indicated by the ammoniacal layer developing a rose pink or cherry red tint.

Balget test:

When sodium picrate is added to the extract, the presence of cardiac glycosides is confirmed by the production of a yellowish-to-orange tint.

Legal's test:

Both sodium nitroprusside and pyridine were used to treat the extracts. When a pink to red color formed, it indicated that cardiac glycosides were present.

Keller Killiani test (for deoxy sugars):

Add two milliliters of glacial acetic acid, one drop of 5% FeCl3, and conc H2SO4 to 0.5 grams of dried extract. A reddish-brown ring that formed indicated the presence of cardenolides.

Detection of Triterpenoids and Sterols:

Liebermann Burchard's test:

After using chloroform, the extracts were combined. Add 1-2 ml of acetic anhydride to the mixture, bring to a boil, and then allow to cool. Conc. H2SO4 was poured into the test tube through the sides. Steroid or triterpenoid saponin glycosides were proven to be present at the junction by the production of brown, pink, blue, and finally green colored rings.

Acetic anhydride was added to the chloroform extract. The top layer of the test tube glowed green when concentrated H2SO4 was added from the side wall, suggesting the presence of steroids.

Detection of Saponins:

Froth's test:

In a graduated cylinder, shake the extracts briskly for 15 minutes with 20 ml of distilled water. The presence of saponins was verified by the production of persistent foam.

Detection of Tannins and Phenolic compounds:

Ferric chloride test:

Add a few drops of 5% neutral ferric chloride solution to the extract. The bluish-black tint that formed indicated the presence of phenolic chemicals.

Vanillin hydrochloric test:

A small amount of vanillin hydrochloride reagent was added to the extracts. Tannins were present because a pinkish-red tint started to appear.

Gelatin test:

A 1% gelatin solution containing NaCl was added to the extract. The presence of tannins was verified by the production of a white precipitate.

Detection of Flavonoids:

Shinoda test:

A small amount of magnesium turning pieces was added to the extracts, and then drops of strong HCl were added. The development of a pink tint indicated the presence of flavonoids.

Lead acetate test:

Add a few drops of 10% lead acetate solution to the extracts. The presence of flavonoids was established by the production of a yellow precipitate.

Alkaline reagent test:

Sodium hydroxide was added in little drops to the extracts. The presence of flavonoids was established by the production of a bright yellow hue that went colorless when a few drops of diluted acid were added.

Detection of Proteins and Amino acids:

Millions test:

Two milliliters of Millon's reagent were combined with the extracts. Proteins and amino acids were demonstrated by the production of a white precipitate that, when heated, turned brick red or when ppt dissolved and produced a red-colored solution.

Biuret test:

Three milliliters of 4% NaOH solution and a few drops of 1% $CuSO_4$ solution were used to treat the extracts. The development of a pink or violet hue indicated the presence of proteins.

Ninhydrin test:

A 5% ninhydrin reagent was added to the extracts and heated for ten minutes. The presence of amino acids was indicated by the creation of a purple or bluish tint.

Detection of Fats and Fixed oils:

Stain test:

Separately, a tiny amount of extracts was pressed between two filter sheets. The presence of fixed oil was verified by an oily spot-on filter paper.

Soap test:

The extracts were heated using 0.5 N alcoholic KOH solutions in a water bath. The existence of fats and fixed oils was demonstrated by the soap's production.

Detection of Carbohydrates:

Each extract was separately diluted in five milliliters of distilled water and then filtered. The presence of carbohydrates was examined using the filtrates.

Molisch’s test:

In a test tube, filtrates were combined with two drops of an alcoholic α-naphthol solution, and two milliliters of concentrated H_2SO_4 were gently added via the test tube's walls. Carbs were present because a violet ring formed at the intersection of two liquids.

Test for reducing sugars

Benedict’s test:

Benedict's reagent was applied to the filters, and they were heated for five minutes in a water bath. Reducing sugars were present because an orange-red precipitate formed.

Fehling's test:

Filtrates were heated with Fehling's A and B solutions, neutralized with alkali, and hydrolyzed with diluted hydrochloric acid. Reducing sugars were present because a brick-red precipitate formed.

Test for monosaccharides:

Barfoed's test:

Barfoed's reagent was added to the filtrates, and they were then cooked in a water bath. The presence of reducing sugars was verified by the observation of red precipitate.

4.4 Phytochemical Analysis of Crude Extract (WHO guidelines, 1998)

To get data on sensory qualities, consistency, and percentage extractive value, phytochemical analysis, both qualitative and quantitative, was performed on all three crude extracts.

4.4.1 Ash Values

a) Determination of Total Ash

In a tared silica crucible, the drug's powder was weighed exactly to the nearest 2g. At the crucible's base, a thin layer of the drug powder was applied. Until the crucible was carbon-free, it was burned at a temperature of no more than 550°C. After cooling, the crucible was weighed.

Until a steady weight was noted, the process was repeated. The air-dried medication was used as a reference to compute the percentage of total ash.

b) Determination of Acid-insoluble Ash

For five minutes, 25 milliliters of hydrochloric acid were boiled with the ash that was collected as shown in the total ash determination. After being filtered using ashless filter paper, the insoluble ash was given a hot water wash. After being moved into a tared silica crucible, the insoluble ash was lit, allowed to cool, and then weighed. Until a steady weight was noted, the process was repeated. With reference to the medication that was air-dried, the proportion of acid-insoluble ash was computed.

c) Determination of Water-soluble Ash

Using 25 milliliters of boiling water, the ash collected as specified in the total ash determination was cooked for five minutes. After gathering the insoluble material on ashless filter paper, hot water was used to wash it away. After being moved into a tared silica crucible, the insoluble ash was lit at a temperature not to exceed 550°C. Until a steady weight was noted, the process was repeated. The weight of the whole amount of ash was deducted from the weight of the insoluble substance. The weight differential was interpreted as water-soluble ash. For medications that were air-dried, the proportion of water-soluble ash was computed.

d) Determination of Sulphated ash:

After being lit for thirty minutes at 600±50ºC, a silica crucible was cooled in a desiccator before being weighed. Take two grams of the powdered medication and wet it with a small amount (about one milliliter) of concentrated sulfuric acid in a precisely weighed crucible. After letting the sample cool and being heated slowly on a low flame until it was completely charred—that is, until no more white fumes emerged—the residue was soaked with concentrated sulfuric acid and heated again. The crucible was set on fire at a temperature of 600±50ºC until the residue was fully burned. After allowing the crucible to cool in a desiccator, it was precisely weighed. carried on the process until the difference between two subsequent weights was not greater than 0.5 mg. Three determinations were recorded, with the average value.

4.4.2 Extractive values (WHO guidelines, 1998)

The nature of a crude drug's ingredients is revealed by its extractive values. Two categories of extractive values exist:

a) Hot percolation:

i) Water-soluble extractive

100 ml of water was added to a conical flask containing 4 g of the medication. After 30 minutes of gentle stirring, this mixture is heated in a water bath. Periodically, the solution was given a little shaking. The mixture was then removed from the water bath, allowed to cool, and then run through a cotton plug filter. A 25 ml portion of the filtrate was removed and dried by evaporating it. To determine the real value, the extract's weight was calculated and multiplied by five.

b) Cold maceration:

i) Ethanol soluble extractive:

A stopper flask was filled with 4 g of previously weighed air-dried medication and 100 ml of 95% ethanol. It was shaken nonstop on a magnetic stirrer for five hours. After that, it was swiftly filtered while being careful not to lose the solvent. In a tared, flat-bottomed petri dish, 25 ml of the filtrate was evaporated till dry, dried at 105°C, and weighed. For medications that were air-dried, the percentage of ethanol-soluble extractive was determined.

ii) Water soluble extractive:

100 ml of water was introduced to a stopper flask containing 4 g of previously weighed air-dried medication. It was shaken nonstop on a magnetic stirrer for five hours. After that, it was quickly filtered while being careful not to lose the solvent. In a tared, flat-bottomed petri dish, 25 ml of the filtrate was evaporated till dry, dried at 105°C, and weighed. For medications that were air-dried, the percentage of water-soluble extractives was determined.

4.4.3 Determination of moisture content

i) Loss on drying:

The method described below was used to substances that seemed to have water as the only volatile element in order to calculate the amount of volatile matter (i.e., water drying off from the medicine).

The drug sample, weighing around 10g, is put on an evaporating dish covered in tar. After being dried at 105°C for six hours, the tarred evaporating dish is weighed once again. The drying process was continued until the variation in weight between subsequent measurements was less than 0.25% of the average weight. With reference to the starting weight, the percentage was computed.

4.4.4 Determination of foreign matter

The original sample, weighed precisely to be between 100 and 500 g, or the amount given in the specific monograph, was spread out in a thin layer. The sample was examined without assistance or with the use of a 6X lens, and every effort was made to manually sort out any foreign biological debris. The weight of the sample was used to calculate the percentage of foreign organic materials.

4.4.5 Foaming index

Saponins found in a variety of medicinal plant materials can produce persistent foaming when an aqueous decoction is agitated. A foaming index is developed to

assess the foaming capacity of plant materials and their extracts when they are decocted aqueously.

Procedure:

The process involved grinding 1 gram of the plant material to a coarse powder (sieve size 1250), measuring it precisely, and then pouring it into a 500 ml conical flask with 100 ml of boiling water within. 30 minutes of moderate boiling were followed by cooling and filtering into a 100 ml volumetric flask. Enough water was then added to the filter to get the volume up to 100 ml.

The aforesaid decoction should be divided into ten test tubes with stoppers, one for each of the numbers 1, 2, 3, and up to 10 ml. The liquid volume in each tube should then be adjusted with water to equal 10 ml. Put a stopper on the tubes and shake them lengthwise twice a second for fifteen seconds. After letting it stand for 15 minutes, measure the height of the foam.

- ✓ The foaming index was less than 100 when the height of the foam in each tube was less than 1 cm.
- ✓ The dilution of the plant material in the tube (a) was the index sought when a 1cm height of foam was measured in any tube.
- ✓ The foaming index was more than 1000 in each tube where the height of the foam exceeded 1 centimeter. To get a result in this instance, a fresh set of decoction dilutions had to be used for the determination.

$$\text{Foaming Index} = 1000/a$$

Where a, is the amount of the decoction in milliliters that was used to prepare the dilution in the tube where foaming was noticed.

4.4.6 Determination of swelling index

The volume in milliliters that one gram of plant material will swell under given conditions is known as the swelling index. The addition of water or a swelling agent, as indicated in the test protocol for each plant material, is what determines it.

Make at least three determinations for a given substance concurrently. Pour the designated amount of the relevant plant material—which has already been precisely weighed and reduced to the necessary fineness—into a 25 ml glass stopper measuring cylinder. The graded part, which is marked in increments of 0.2 milliliters from 0 to 25 milliliters in an upward orientation, should have an internal diameter of approximately 16 mm and a length of about 125 milliliters. Add 25 ml of water, and

shake the mixture well every 10 minutes for an hour, unless directed otherwise in the test protocol. Let remain at room temperature for three hours, or as directed. Determine the total volume in milliliters that the plant material—including any sticky mucilage—occupies. Determine the average value of each determination in relation to one gram of plant material.

4.4.7 Determination of solvent residue

The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) defines residual solvents in pharmaceutical products as "organic volatile chemicals that are used or produced in the manufacture of drug substances or excipients, or in the preparation of drug products (ICH Q3C(R8) 2021).

4.4.7.1 Sample preparation:

Accurate amount of each of the extracts (CMME, TUME, and SIME) into the vial and seal it with a crimp cap.

4.4.7.2 Parameters of GC-MS and Headspace for Solvent Residue Analysis:

Table 4.1: Parameters of GC-MS and Headspace for Solvent Residue Analysis

Sr. No	GCMS details	
1	**GC-MS Model:**	TQ8040
2	**Software of Mass Spectra and Chromatogram:**	Lab Solution Version 5.91
3	**Column Type:**	Capillary column
4	**Column Material:**	95% Dimethylpolysiloxane
5	**Column Length:**	30 meters
6	**Column Inner Diameter:**	0.320 mm
7	**Programming Rate:**	Starting from 75 ℃ for 5min. Increasing Temperature with rate 10 ℃/min up to 280 ℃ and hold for 20 min.
8	**Temperature of Source:**	220 ℃
9	**Temperature of Injector:**	270 ℃
10	**Temperature of Detector:**	280 ℃

11	**Temperature of Transfer:**	250 ℃
12	**Carrier Gas:**	Helium
13	**Injection Volume:**	1 microlitre
14	**Flow Rate:**	1.2 ml/min
15	**Scan Range:**	20 to 620 amu, at 70eV
16	**Retention Time:**	52 min.
17	**Detector**	Mass spectrometer
Head Space Condition		
1	**Oven Temperature**	60℃
2	**Sample Line Temperature**	150℃
3	**Transfer Line Temperature**	150℃
4	**GC Cycle time**	52 min
5	**Injection Time**	1 min
6	**Equilibrating Time**	20 min
7	**Pressurizing time**	2 min
8	**Pressure equilibration time**	0.10 min
9	**Load time**	1 min
10	**Load Equilib. Time**	0.10 min
11	**Pressurization time**	2 min
12	**Vial Equilibration time**	18 min
13	**Pressurizing Gas pressure**	50 kpa
14	**Needle Flush Time**	5 min

The TQ8040 was coupled with a multipurpose autosampler. A 1 mL aliquot of headspace was injected at 200 $\mu L \cdot s^{-1}$ into the spitless inlet of the GC. Separation was achieved using a column (30 m × 0.32 mm × 1.8 μm; Agilent Technologies, Santa Clara, CA, USA) with a helium flow rate of 2.15 $mL \cdot min^{-1}$. The oven was initially held at 50 °C for 5 min before a ramp of 4 $°C \cdot min^{-1}$ to 150 °C followed by ramp of 30$°C \cdot min^{-1}$ to 260 °C, where it was held for 5 min. Eluting components were detected using a mass detector operating at 270°C with helium gas as mobile phase at 1.9 ml/min flowrate.

4.5 Determination of Total phenolic content of crude extract

Wolfe et al. (2003) described a modified Folin-Ciocalteu method for determining the total phenolic contents in the extracts.

Principle:

The Folic-Ciocalteu reagent reacts with phenolic chemicals to generate a blue color complex that absorbs light at 760 nm, which is the basis for this assay.

Reagents:

- Folin-Ciocalteu reagent: dilution made 1:10 v/v before use.
- Sodium carbonate (75 g/L)
- A stock solution of plant extracts is 1mg/ml

Procedure:

A 2.0 ml of Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 2.0 ml (75 g/L) of sodium carbonate were combined with an aliquot of all three plant crude extracts. To develop the color, the tubes were vortexed for 15 seconds and then let to stand at 25°C for 20 minutes. Using various amounts of tannic acid as reference standard (10, 20, 30, 40, and 500 µg/ml), a standard curve was constructed. Next, absorbance was determined using a UV spectrophotometer set to 760 nm. For every measurement, duplicate samples were generated, and the calibration curve was plotted using the average absorbance value to ascertain the extracts' phenolic content. The total phenolic contents were expressed in terms of tannic acid equivalents (TAE).

4.6 Determination of Total Flavonoid Content of crude extract

We evaluated total flavonoids by applying the Ordonez et al. (2006) approach.

Principle:

Aluminum chloride ($AlCl_3$) forms acid-stable complexes with C-4 keto groups and either C-3 or C-5 hydroxyl groups of flavones and flavanols. It also forms acid labile complexes with Ortho dihydroxyl groups in the A- or B- ring of flavonoids.

Reagents:

- Methanol
- 10 % Aluminum chloride
- 1 M Potassium acetate
- A stock solution of plant extracts is 1 mg/ml

Procedure:

0.5 ml of sample (all three plant crude extracts) was mixed with 1.5 ml of methanol, 100 µl of 10% aluminum chloride, 100 µl of 1 M potassium acetate solution, and 2.8 ml of distilled water. After incubating at room temperature for 1.5 hours, the absorbance at 420 nm was measured. Quercetin Equivalent (QUE) milligrams were used to quantify the total flavonoid content, and reference standards with different quercetin concentrations in methanol (20, 40, 60, 80, and 100 µg/ml) were used to create a standard curve.

4.7 Determination of Total Antioxidant Activity (TAC) of crude extract

With minor adjustments, the technique described by Prieto et al. (1999) was used to calculate the TAC of the samples.

Principle:

The phosphomolybdanum ion is reduced from Mo (VI) to Mo (V) in the presence of an antioxidant, leading to the creation of a green phosphate/MoV complex, which has maximum absorption at 695 nm in acidic circumstances. This is the basis for the assay.

Reagents:

- 0.6 M Sulphuric acid
- 28 mM Sodium phosphate
- 1% Ammonium molybdate

Procedure:

The test tubes were filled with 3.0 ml of reaction mixture that contained 0.6 M sulfuric acid, 28 mM sodium phosphate, and 1% ammonium molybdate. Additionally, 0.5 ml of extracts/fractions that contained 500 µg of extract were added. The test tubes were incubated at 95 °C for 10 minutes to complete the operation. Using a spectrophotometer and a suitable blank, the absorbance was measured at 695 nm once the sample had cooled to room temperature. A standard curve of ascorbic acid was plotted using the same procedure at different concentrations (25, 50, 100, 200, and 300 µg/ml). mg AAE was employed to convey the findings.

4.8 *In-vitro* anticoagulant activity of crude extracts

4.8.1 Experimental animals

Sprague Dawley rats with a weight range of 180 to 220 grams were sourced from the animal house facility. These rats were housed in ventilated rooms maintained at a temperature of 24 ± 2 degrees Celsius, with a 12-hour light-dark cycle and a relative humidity of 54 ± 5%. Throughout the experiment, they were fed regular pellet food and had unlimited access to water. The animals underwent a one-week habituation period. The experiments were conducted per the guidelines established by the CCSEA and were approved by the IAEC (Institutional Animal Ethical Committee) (BKMGPC/IAEC28/RP91/2022) of B. K. Mody Government Pharmacy College, Rajkot, Gujarat, India.

4.8.2 Acute toxicity

Based on a review of the literature, the acute toxicity doses for CMME (*Citrus medica* Methanolic Extract), TUME (*Tecomella undulata* Methanolic Extract), and SIME (*Sesamum indicum* methanolic Extract) have been identified as 5000 mg/kg (Ningombam et.al 2021, Goyal et.al 2010, Yusuf et.al 2017).

4.8.3 Blood Sampling and plasma preparation

Venous blood was taken straight out of healthy rats and utilized to calculate the clotting time. Venous blood was drawn for the invitro anticoagulant assay in 1:10 (v/v) 3.8% trisodium citrate. Then, using a Compact Centrifuge, the citrated blood samples were centrifuged at 6000 rpm for 10 minutes in order to extract platelet-poor plasma (PPP) (Shah et al., 1999). Before being used, the plasma was separated and kept in a refrigerator at -4 °C.

4.8.4 Blood clotting time measurement

The measurement of in vitro clotting time was performed using a modified method based on Lee and White, as previously reported by (Osoniyi and Onajobi, 2003). Clotting tubes were prepared, containing 0.5 ml of various fractions of *Citrus medica* suspended in Normal Saline (NS) at concentrations ranging from 2.5 to 10 mg/mL. There was also a control tube that contained just NS. After that, these tubes were kept at 37 °C in a water bath. After incubation, 0.5 ml of freshly drawn blood was carefully introduced into each of the incubated tubes by allowing it to flow down the side of the tube, while simultaneously starting a stopwatch. At 30-second intervals, the tubes were gently tilted to an angle of 45° to observe the formation of a blood clot. When the first clot was observed, the time it took was noted, and the tilting procedure was repeated until the tubes could be turned upside down without blood leaking out. The

stopwatch was immediately stopped, and the time was recorded as the final clotting time.

4.8.5 Prothrombin time (PT) activity assay

The PT assays were conducted using commercially available reagent kits (PT (Prothrombin time) ®, Robonik India Pvt. Ltd., India) following the recommended protocols provided by the manufacturer (Gao et. al., 2014). For each assay, 100 µl of plasma sample was mixed with 100 µl of various fractions (ranging from 50 to 500 µg/µl) that were diluted in normal saline. After a 5-minute pre-incubation period, 100 µl of PT assay reagent (consisting of rabbit brain extract and calcium chloride) pre-warmed at 37°C for 10 minutes was added, and the clotting time was recorded and measured. A vehicle control using normal saline was used for comparison. Each PT assay was performed in triplicate.

4.8.6 Activated Partial Thromboplastin time (APTT) activity assay

APTT activity assays were conducted using commercially available reagent kits (APTT (Activated prothrombin time testing) ®, Robon 6t ik India Pvt. Ltd., India) following the recommended protocols provided by the manufacturer (Gao et. al., 2014). For each assay, 100 µl of plasma was mixed with 100 µl of various fractions (ranging from 50 to 500 µg/µl) at 37°C. After a 5-minute pre-incubation period, pre-warmed APTT reagent was added to the mixture. The clotting time was measured after the addition of pre-warmed 50 µl of CaCl2 solution. Normal saline was used as a vehicle control. The APTT assay was performed in triplicate.

4.8.7 Statistical Analysis:

The data were presented as means ± SD based on three measurements (n=3). Statistical comparisons of the extended time among all extracts and the vehicle control (0.9% Normal Saline) were performed using one-way ANOVA (Multiple comparisons) with GraphPad Prism version 6.0 (San Diego, California). A p-value greater than 0.01 was considered statistically significant.

4.9 Preparation of fractions

Fractionation was carried out in accordance with the Kupchan-designed protocol that van Wagenen et al. (1993) modified.

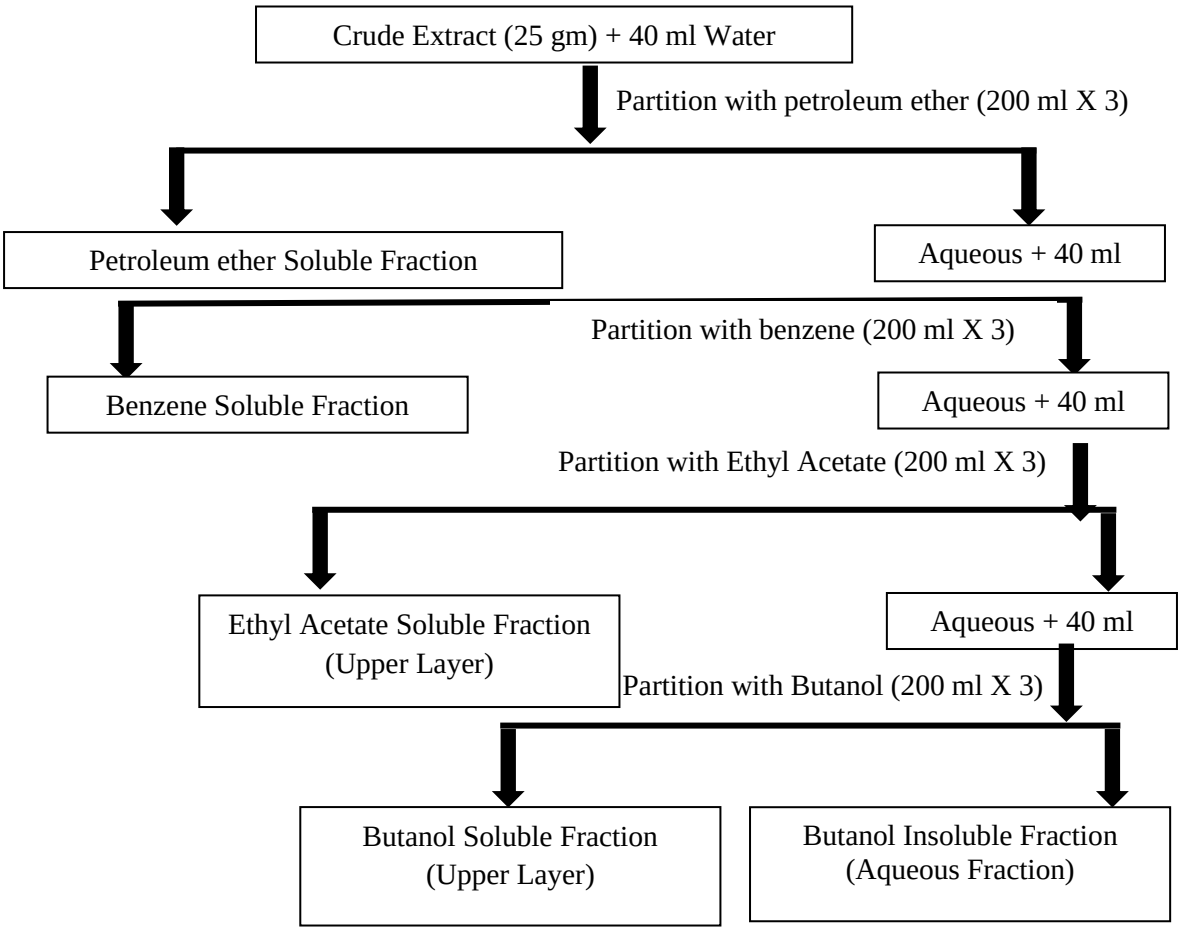

Figure 4.4: Schematic representation of modified Kupachan partitioning

As illustrated in figure 4.4, TUME and CMME (25 g) were exposed to solvent-solvent sequential partitioning using solvents of increasing polarity (3×200 ml for each solvent type): petroleum ether, benzene, ethyl acetate, and butanol. Petroleum ether fraction (PF), benzene fraction (BF), ethyl acetate fraction (EAF), and butanol fraction (BUF) were obtained by evaporating the combined solvent component of the corresponding fractions. 40 cc of distilled water were introduced at each stage of the solvent fractionation process. Residues insoluble in butanol were regarded as aqueous fraction (AF). At a low temperature of 40 oC, all fractions were evaporated until dry and then kept until needed again.

4.10 Preliminary phytochemical screening of fractions

A qualitative phytochemical screening was performed on all TUME and CMME fractions to check for the presence of different secondary metabolites (Khandelwal KR 2009).

4.10.1 Materials

Test tubes, Soxhlet apparatus, desiccators, digital electronic weighing instruments, distillation apparatus, chromatographic paper, and TLC plates were used. All the chemicals are of Analytical Grade Reagent of Angel Tradelink, Rajkot, Gujarat.

4.10.2 Preliminary phytochemical screening (Kokate, 2001; Khandelwal, 2001)

The procedure follows as per 3.3.2

4.11 Phytochemical analysis of fractions

The procedure follows as per 3.4.

4.12 Determination of Total Phenolic content of fractions

The procedure follows as per 3.5.

4.13 Determination of Total Flavonoid content of fractions

The procedure follows as per 3.6.

4.14 Determination of Total Antioxidant capacity of fractions

The procedure follows as per 3.7.

4.15 *In-vitro* antioxidant activity of fractions of potent crude extract of the plant

4.15.1 DPPH Radical scavenging activity:

The DPPH radical scavenging assay (1, 1-Diphenyl-2-Picrylhydrazyl) was used to examine the extracts' capacity to scavenge free radicals, following the protocol outlined by Choi et al (2000).

Principle:

DPPH radical is scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The decrease in absorbance at wavelength 517 nm following reduction can be used to measure the color change from purple to yellow.

Reagents:

→ 0.2 mM DPPH

→ 80% Methanol

Procedure:

A mixture of 1.6 ml of different fraction concentrations (25, 50, 75, 100, 150, 200, and 250 µg/ml) and 2.4 ml of methanolic DPPH solution (0.1 mM) was prepared. After giving the mixture a good shake, it was allowed to sit at room temperature for half an hour in the dark. Using spectrophotometry, the mixture's absorbance was calculated at 517 nm. An ascorbic acid reference control was employed. Ascorbic acid concentrations (10, 25, 50, 100, and 200 µg/ml) were all treated in the same way. The equation was used to determine the proportion of free radical scavenging activity (% DRSA), which was demonstrated by the staining of DPPH.

$$\% \text{ FRSA} = [(A_0 - A_1)/ A_0] \text{ X } 100$$

Where A_0 is the control's absorbance, and

A_1 is the extracts/standard's absorbance

4.15.2 Superoxide radical scavenging activity:

The test plant's crude extracts and fractions were tested for their ability to scavenge superoxide radicals using a modified version of the Chun, O.K. et al. 2003 method.

Principle:

By detecting the intensity of blue color at 570 nm, the experiment was predicated on the extracts' ability to suppress the formation of blue-colored nitro-blue tetrazolium formazan (NBT2+) by scavenging the superoxide radicals produced in the riboflavin-light-NBT system.

Reagents:

→ M EDTA

→ 0.0015 % NaCN

→ 0.12 mM riboflavin

→ 1.5 mM nitro blue tetrazolium (NBT)

→ 67 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.8

Procedure:

In addition to various fractions (25, 50, 75, 100, 150, 200, and 250 µg/ml), phosphate buffer (67 mM, pH 7.8), 200 µl NaCN, 50 µl riboflavin (0.12 mM), and 100 µl NBT (1.5 mM) were included in the reaction mixture, which had a total volume of 3 ml. The optical density at 530 nm was determined after the tubes were continuously illuminated for 15 minutes. Ascorbic acid was used in the same technique at different concentrations: 25, 50, 100, 200, and 300 µg/ml. To determine the percentage of inhibition, calculations were made. A parallel blank

was carried out, with distilled water used in place of the sample in the reaction mixture.

$$\% \text{ Inhibition} = [(A_0 - (A_1 - A_2)/ A_0] \text{ X } 100$$

Where A_0= Blank absorbance, A_1= sample absorbance,

A_2= Sample absorbance without NBT

4.15.3 Nitric oxide radical scavenging activity:

At physiological pH, sodium nitroprusside solution produced nitric oxide radicals (Jayaprakasha et al 2004).

Principle:

The Greiss technique is the basis of the assay. Diazotization of sulphanilamide could result from the spontaneous generation of NO in an aqueous solution of sodium nitroprusside at physiological pH interacting with oxygen to produce nitrite ions. When naphthylethylenediamine dichloride and the diazotized product combine, an azo-dye intensity is formed, which is measured at 550 nm.

Reagents:

→ 10 mM Sodium nitroprusside

→ 0.25 M Phosphate buffer saline (PBS, pH 7.4)

→ 1.0 % Sulphanilic acid reagent: prepared in 2 % orthophosphoric acid

→ % Naphthylethylenediamine (NEDD)

Procedure:

Each test fraction (25, 50, 75, 100, 150, 200, and 250 µg/ml) in PBS (pH 7.4) was divided into four milliliters and mixed with ten milliliters of sodium nitroprusside (10 mM). The mixture was incubated at 25 °C for 150 minutes. After thoroughly mixing 1.0 ml of the 1% sulphanilic acid reagent (in 2% orthophosphoric acid) with 0.5 ml of the incubated solution, the mixture was allowed to stand for 5 minutes in order to achieve complete diazotization. Then, 1.0 milliliter of 0.1% NEDD (naphthylethylenediamine) was added, shaken, and the mixture was exposed to darkness for half an hour to observe the formation of a pink chromophore. Different quantities of ascorbic acid (25, 50, 100, 150, and 200 µg/ml) were used in the same process. These solutions' absorbance was measured at 540 nm in relation to a blank solution that had PBS instead of a sample. The proportion of nitric oxide radical scavenging was calculated using the equation.

$$\% \text{ Scavenging} = [(A_0 - A_1)/ A_0] \times 100$$

Where A_0 is the control's absorbance, and

A_1 is the extracts/fractions/standard's absorbance

4.15.4 Metal ion chelating activity:

Gulcin (2006) devised a method for estimating the ability of extracts to chelate iron ions.

Principle:

The assay is based on the idea that ferrozine and free ferrous ion (Fe2+) combine to form a pink-purple complex. Any material that obstructs the formation of the Ferrozine-Fe2+ complex results in a decrease in color intensity, which is measured at 562 nm.

Reagents:

→ Methanol

→ 2 mM $FeCl_3$

→ 5 mM ferrozine in methanol

Procedure:

Several concentrations of ascorbic acid (25, 50, 100, 200, and 300 µg/ml) and the fractions (25, 50, 75, 100, 150, 200, and 250 µg/ml) were combined with 2.5 ml of 2 mM FeCl3. After adding 4 ml of methanol, the mixture was quickly stirred and allowed to stand at room temperature for 10 minutes. 5 mM ferrozine was added in 0.2 ml increments to initiate the reaction. After an incubation period, spectrophotometry was used to measure the color at a wavelength of 562 nm.

The percentage of inhibition was calculated using the formula:

$$\% \text{ inhibition} = [A_0 - (A_1 - A_2)]/A_0 \times 100$$

Where A_0 is the control's absorbance, containing $FeCl_3$ and ferrozine only,

A_1 is the sample's absorbance

A_2 is the Sample's absorbance under the same circumstances as A1, but using methanol rather than ferric chloride solution.

EDTA was used as a standard.

4.15.5 Reducing power:

The method provided by He, N. et al. (2012) was modified to determine the reducing power of test extracts/fractions.

Using Oyaizu's approach, the Fe3+ reducing power of various fractions was calculated. This approach involved measuring Pearl's Prussian blue complex

absorbance to assess the decrease of Fe3+ to Fe2+. The reduction of (Fe3+) ferricyanide in stoichiometric excess in relation to the antioxidants is the basis of this technique.

Reagents:

→ 0.2 M Phosphate buffer (pH 6.6)

→ 1 % Potassium ferricyanide [$K_3Fe(CN)_6$]

→ 10 % Trichloroacetic acid (TCA)

→ 0.1 % Ferric chloride solution

Procedure:

Apart from ascorbic acid (25, 50, 100, 200, and 300 µg/ml) and fractions with varying concentrations (5, 10, 25, 50, 75, 100, and 125 µg/ml), 2.5 ml of potassium ferricyanide [K3Fe(CN)6] (1%, w/v) and phosphate buffer (0.2 M, pH 6.6) were mixed. The resultant mixture was incubated at 50°C for 20 minutes. The mixture was centrifuged for 10 minutes at 3000 rpm after 2.5 ml of a 10% TCA solution was added to stop the reaction. The supernatant was then collected. 2.5 ml of supernatant, 2.5 ml of distilled water, and 0.5 ml of ferric chloride solution (0.1%, w/v) were added after thoroughly mixing. The absorbance of the greenish-blue chromogen was measured at 700 nm. The greater reduction was indicated by the reaction mixture's higher absorbance. 2.5 ml of distilled water was used in place of the sample in a parallel blank run.

4.16 Qualitative HPTLC Fingerprinting:

4.16.1 Selection of plate and adsorbent:

The 10 × 10 cm TLC plates were precoated with 0.2 mm thick silica gel 60 F254 TLC plates (E. Merck) and supported by an aluminum sheet. The experimental conditions were maintained at an ideal temperature of 25 ± 2 °C and relative humidity of 40% throughout. The spotting device was a CAMAG LINOMAT V automatic sample spotter (Camag Muttenz, Switzerland); the syringe was 100 µl (from Hamilton); the developing chamber was a CAMAG glass twin trough chamber (20 × 10 cm); the densitometer was a CAMAG TLC Scanner connected to WINCATS software.

4.16.2 Preparation of sample solutions:

In order to effectively extract the marker chemicals and obtain satisfactory fingerprinting, the preparation of sample solutions was adjusted. The chosen

fractions' sample preparation is listed below. Ten milligrams of Tecomella undulata and Citrus medica were precisely weighed into five milliliters of methanol to create each fraction. The final volume of the solutions was increased to 10 ml in a volumetric flask after they were filtered through Whatman I filter paper. After that, fingerprints were taken using these fraction solutions.

4.16.3 Application of samples:

On a TLC plate, 10 µl of a suitably pre-diluted sample solution including all fractions of Citrus medica (CMPEF, CMBF, CMEAF, CMBUF, CMWF) and Tecomella undulata (TUPEF, TUBF, TUEAF, TUBUF, TUWF) was applied. As previously stated, the plates were developed and scanned. The areas of the peak were noted.

4.16.4 Solvent system:

After much trial and error, the solvent systems that produced the most bands overall were determined to be the following ones. After much trial and error, toluene was chosen as the solvent system: Formic acid: ethyl acetate (07:03:0.5) produced the greatest number of bands.

4.16.5 Optimization of Chromatographic Condition:

Table 4.2: Optimization of Chromatographic condition

Stationary phase:	Silica gel 60 F254 TLC Plate
Scanner:	CAMAG TLC scanner 3 and WINCATS 4 software
Developing chamber:	CAMAG glass twin through chamber (20x10 cm)
Chamber saturation:	20 minutes
Distance between tracks:	10 mm
Rate of spotting:	5 µl/sec
Distance run:	85 mm
Scanning speed:	20 mm/sec
Slit dimension:	4 x 0.3 mm, Micro
Temperature:	25 °C
Relative Humidity:	40%
Scanning Wavelength:	255 and 365 nm

Spraying Reagent:	None
Derivatization temperature:	105 for 10 min

4.16.6 Detection:

The plate was scanned at 255 and 366 nm using CAMAG TLC Scanner-3 and LINOMAT-V. The rf value and peak area of each compound separated on the plate were recorded.

4.16.7 Evaluation of TLC plate:

The plates were documented with their photographic measurement using WINCATS software and/or for qualitative evaluation.

4.16.8 Interpretation of HPTLC chromatograph:

Qualitative evaluation is used for HPTLC fingerprinting of different fractions which gives ideas about the presence or absence of one more compound in different fractions or in single fractions.

4.17 *In-vitro* anticoagulant activity of fractions

4.17.1 Experimental animals

Sprague Dawley rats with a weight range of 180 to 220 grams were sourced from the animal house facility. These rats were housed in ventilated rooms maintained at a temperature of 24 ± 2 degrees Celsius, with a 12-hour light-dark cycle and a relative humidity of 54 ± 5%. They were provided with standard pellet food and had access to water ad libitum throughout the experimental period. The animals underwent a one-week habituation period. The experiments were conducted per the guidelines established by the CCSEA and were approved by the IAEC (Institutional Animal Ethical Committee) (BKMGPC/IAEC28/RP91/2022) of B. K. Mody Government Pharmacy College, Rajkot, Gujarat, India.

4.17.2 Blood sampling and plasma preparation

Venous blood was directly collected from healthy rats and used for the clotting time measurement. Venous blood was collected in 1:10 (v/v) 3.8% trisodium citrate for invitro anticoagulant assay. The citrated blood samples were then subjected to centrifugation at 6000 rpm for 10 minutes using a Compact

Centrifuge to obtain platelet-poor plasma (PPP) (Shah et. al., 1999). The plasma was separated and stored in the refrigerator at −4 °C until use.

4.17.3 Blood clotting time measurement

The measurement of in vitro clotting time was performed using a modified method based on Lee and White, as previously reported by (Osoniyi and Onajobi, 2003). Clotting tubes were prepared, containing 0.5 ml of various fractions of *Citrus medica* suspended in Normal Saline (NS) at concentrations ranging from 2.5 to 10 mg/mL. A control tube with NS alone was also included. These tubes were then incubated in a water bath at 37 °C. After incubation, 0.5 ml of freshly drawn blood was carefully introduced into each of the incubated tubes by allowing it to flow down the side of the tube, while simultaneously starting a stopwatch. At 30-second intervals, the tubes were gently tilted to an angle of 45° to observe the formation of a blood clot. The time taken for the first observation of clot formation was recorded, and the tilting process was continued at intervals until the tubes could be inverted without blood flowing out. The stopwatch was immediately stopped, and the time was recorded as the final clotting time.

4.17.4 Prothrombin time (PT) activity assay

The PT assays were conducted using commercially available reagent kits (PT (Prothrombin time) ®, Robonik India Pvt. Ltd., India) following the recommended protocols provided by the manufacturer (Gao et. al., 2014). For each assay, 100 µl of plasma sample was mixed with 100 µl of various fractions (ranging from 50 to 500 µg/µl) that were diluted in normal saline. After a 5-minute pre-incubation period, 100 µl of PT assay reagent (consisting of rabbit brain extract and calcium chloride) pre-warmed at 37°C for 10 minutes was added, and the clotting time was recorded and measured. A vehicle control using normal saline was used for comparison. Each PT assay was performed in triplicate.

4.17.5 Activated prothrombin time (APTT) activity assay.

APTT activity assays were conducted using commercially available reagent kits (APTT (Activated prothrombin time testing) ®, Robon 6t ik India Pvt. Ltd., India) following the recommended protocols provided by the manufacturer (Gao et. al., 2014). For each assay, 100 µl of plasma was mixed with 100 µl of various fractions (ranging from 50 to 500 µg/µl) at 37°C. After a 5-minute pre-incubation

period, pre-warmed APTT reagent was added to the mixture. The clotting time was measured after the addition of pre-warmed 50 µl of CaCl2 solution. Normal saline was used as a vehicle control. The APTT assay was performed in triplicate.

4.17.6 Statistical Analysis:

The data were presented as means ± SD based on three measurements (n=3). Statistical comparisons of the extended time among all extracts and the vehicle control (0.9% Normal Saline) were performed using one-way ANOVA (Multiple comparisons) with GraphPad Prism version 6.0 (San Diego, California). A p-value greater than 0.01 was considered statistically significant.

4.18 *In-vivo* anticoagulant test of Fractions

4.18.1 Experimental animals

Sprague Dawley rats with a weight range of 180 to 220 grams were sourced from the animal house facility. These rats were housed in ventilated rooms maintained at a temperature of 24 ± 2 degrees Celsius, with a 12-hour light-dark cycle and a relative humidity of 54 ± 5%. They were provided with standard pellet food and had access to water ad libitum throughout the experimental period. The animals underwent a one-week habituation period. The experiments were conducted per the guidelines established by the CPCSEA and were approved by the IAEC (Institutional Animal Ethical Committee) (BKMGPC/IAEC30/RP105/2023) of B. K. Mody Government Pharmacy College, Rajkot, Gujarat, India.

4.18.2 Experimental Design (Lei, L et al 2015)

The animal was divided into five groups and each group contained 6 Sprague Dawley rats. Initially, the standard group was treated with Warfarin (0.2 mg/kg, p.o) for 3 days. High, medium, and low doses of potent fractions (CMEAF, TUBUF) of both plant extracts were treated orally with 100, 200, and 400mg/kg for 3 days. The normal group was treated with normal saline for 3 days. To evaluate the in vivo anticoagulant activity of fractions of both plant extracts, the APTT, PT, Platelet count, and clotting time were determined on the 3rd, 5^{th}, and 7th day after First treatment.

Table 4.3: Detail Experimental design method

Sr.No	Group	Treatment Received
1	Normal Control	Saline (p.o)

2	Standard Control	Warfarin (0.2 mg/kg, p.o) for 3 days
3	CMEAF	(100 mg/kg, p.o) in vehicle for 3 days
4	CMEAF	(200 mg/kg, p.o) in vehicle for 3 days
5	CMEAF	(400 mg/kg, p.o) in vehicle for 3 days
6	TUBUF	(100 mg/kg, p.o) in vehicle for 3 days
7	TUBUF	(200 mg/kg, p.o) in vehicle for 3 days
8	TUBUF	(400 mg/kg, p.o) in vehicle for 3 days

CMEAF- *Citrus medica* ethyl acetate fraction, TUBUF – *Tecomella undulata* butanol fraction

4.18.3 Blood sampling collection and plasma preparation:

Venous blood was directly collected from healthy rats by retro-orbital method on the 3rd, 5th, and 7th days after the first treatment and used for the clotting time measurement. Venous blood was collected in 1:10 (v/v) 3.8% trisodium citrate for invitro anticoagulant assay. The citrated blood samples were then subjected to centrifugation at 6000 rpm for 10 minutes using a Compact Centrifuge to obtain platelet-poor plasma (PPP) (Shah et. al., 1999). The plasma was separated and stored in the refrigerator at −4 °C until use.

4.18.4 Statistical Analysis:

The data were presented as means ± SD based on three measurements (n=3). Statistical comparisons of the extended time among all extracts and the vehicle control (0.9% Normal Saline) were performed using one-way ANOVA (Multiple comparison) with GraphPad Prism version 6.0 (San Diego, California). A p-value greater than 0.01 was considered statistically significant.

4.19 GC-MS Analysis:

4.19.1 Preparation of the fraction:

Accurately weighing 10 mg in 5 ml of methanol produced the butanol fraction of *Tecomella undulata* fraction (TUBUF) and the ethyl acetate fraction of *Citrus medica* (CMEAF). In order to eliminate the sediments and any remaining water from the filtrate, the fractions were filtered using Whatman I filter paper and two

grams of sodium sulfate. Methanol was used to moisten the filter paper and sodium sulfate before filtering. Both polar and non-polar phytocomponents of the utilized plant material were present in the extract. For the GC/MS analysis, one microliter of these solutions was used (Merlin N et al., 2009).

4.19.2 Parameters of GC-MS Analysis:

Toshvin TQ8040 GC-MS model, Perkin Elmer Autosystem XL with Lab Solution Version 5.91, PE-5MS column type, 5% phenyl polysiloxane column material, 30 m column length, 0.250 mm inner diameter, and 1 ml/min flow rate Temperatures of the injector are 250 °C, detector is 280 °C, source is 280 °C, transfer is 280 °C, and programming rate is 78 °C for five minutes. raising the temperature to 280°C in 20 minutes at a pace of 10°C per minute. 45 minutes of retention.

4.19.3 Optimization parameter of GC-MS analysis Conditions:

Table 4.4: Optimization parameter of GC-MS

Sr. No	GCMS details	
1	**GC-MS Model:**	TQ8040
2	**Software of Mass Spectra and Chromatogram:**	Lab Solution Version 5.91
3	**Column Type:**	Capillary column
4	**Column Material:**	100% Dimethylpolysiloxane
5	**Column Length:**	30 meters
6	**Column Inner Diameter:**	0.320 mm
7	**Programming Rate:**	Starting from 75 ℃ for 5min. Increasing Temperature with rate 10 ℃/min up to 280 ℃ and hold for 20 min.
8	**Temperature of Source:**	220 ℃
9	**Temperature of Injector:**	270 ℃
10	**Temperature of**	280 ℃

	Detector:	
11	**Temperature of Transfer:**	250 ℃
12	**Carrier Gas:**	Helium
13	**Injection Volume:**	1 microlitre
14	**Flow Rate:**	1.2 ml/min
15	**Scan Range:**	20 to 620 AMU, at 70eV
16	**Retention Time:**	72 min.

4.19.4 Identification of components:

To obtain the spectrum data, separated chromatograms of several phytoconstituents were subsequently subjected to mass fragmentation. The National Institute of Standard and Technology (NIST) database, which contains more than 62,000 patterns, was used to interpret the GC-MS analytical spectrum. A comparison was made between the mass spectrum of the known components kept in the NIST library and the unknown components of TUBUF and CMEAF. We determined the components of the test extract fractions, including their names, molecular weights, and structures.

4.20 LCMS/MS Analysis:

4.20.1 Preparation of the fractions:

Accurately weighing 10 mg in 5 ml of methanol produced the butanol fraction of *Tecomella undulata* fraction (TUBUF) and the ethyl acetate fraction of *Citrus medica* (CMEAF). Whatman I filter paper was used to filter the fractions, and 2 g of sodium sulfate was added to the filtrate to eliminate any sediments and remaining water. Methanol was used to moisten the filter paper and sodium sulfate prior to filtering. Both polar and non-polar phytocomponents of the utilized plant material were present in the extract. For the LCMS/MS analysis, 1µl of these solutions was used (Zahoor, M., et al).

4.20.2 Preparation of Standard Stock Solution

- **Quercetin Stock Solution, 1mg/mL**

The final concentration of quercetin was adjusted to 1 mg/mL by adding an appropriate volume of methanol, considering both the potency and the actual

amount weighed. The quercetin standard was precisely weighed to equal 10 mg of quercetin. The solution was kept at 5±3°C in a refrigerator. After the preparation date, use the solution within seven days.

- **Rutin Stock Solution, 1mg/mL**

  A suitable volume of methanol was added to the precisely weighed Rutin standard to bring the final concentration of Rutin to 1 mg/mL, considering both its potency and the exact amount weighed. The solution was kept at 5±3°C in the refrigerator. After the preparation date, use the solution within seven days.

- **Gallic acid Stock Solution, 1 mg/mL**

  A suitable volume of methanol was added to the precisely weighed 10 mg of gallic acid standard to achieve a final concentration of 1 mg/mL, which takes into consideration both the acid's potency and the exact amount weighed. The solution was kept at 5±3°C in the refrigerator. After the preparation date, use the solution within seven days.

4.20.3 Mix Drug intermediate Solution (100µg/mL of each standard):

In a 10.0 mL volumetric flask, pipette out 1000.0µL of the 1 mg/mL Quercetin, Rutin, Naringin, and Gallic acid stock solution. Add methanol to bring the volume up to 10.0 mL. The solution was kept at 5±3°C in the refrigerator. After the preparation date, use the solution within seven days.

4.20.4 Solvent system

0.2% Formic acid in water

Aaccurately transfer 2.0 mL of formic acid into 1000 mL of HPLC-grade water, bringing the total volume to 1000 mL. moved into a bottle of reagent. kept in room temperature storage. The day of preparation was followed by three days of use for this solution.

4.20.5 Auto Sampler Rinsing Solution

500 milliliters of methanol and 500 milliliters of water were taken out of the measuring cylinder, put into a reagent bottle, and properly mixed. kept in a room temperature storage. The day of preparation was followed by three days of use for this solution.

4.20.6 Calibration Curve (CC) Spiking Solutions:

CC Spiking solutions were prepared in Methanol using Mix Drug Intermediate Solution (Quercetin, Rutin, and Gallic acid are 100µg/mL) by serial dilution as described below:

Table 4.5: Spiking solution of standards for the calibration curve

Stock Dil. Conc. QUE (ng/mL)	**Stock Dil. Conc. RUTIN (ng/mL)**	**Stock Dil. Conc. GA (ng/mL)**	**Vol. Taken (mL)**	**Vol. of methanol (mL)**	**Total Vol. (mL)**	**Spiking Solution Conc. All standards (ng/mL**	**Spiking Solution ID**
100000	100000	100000	0.500	1.500	2.000	25000	SS STD1
25000	25000	25000	1.600	0.400	2.000	20000	SS STD2
20000	20000	20000	1.000	1.000	2.000	10000	SS STD3
10000	10000	10000	1.000	1.000	2.000	5000	SS STD4
5000	5000	5000	1.000	1.000	2.000	2500	SS STD5
2500	2500	2500	1.000	1.000	2.000	1250	SS STD6
1250	1250	1250	0.800	1.200	2.000	500	SS STD7
500	500	500	0.800	1.200	2.000	200	SS STD8

4.20.7 LC-MS/MS instrumentation and Chromatographic condition

Table 4.6: LC-MS/MS instrumentation

Sr. No.	**Instrument**	**Brand / Model**	**Manufacturer / Supplier**
1.	Auto-sampler	Shimadzu SIL-20AC	Shimadzu

2.	Column oven	CTO-20AC	Shimadzu
3.	Pump	LC-20ADvp	Shimadzu
4.	Degasser	DGU-20A5R	Shimadzu
5.	System Controller	CBM-20A SPD-M20A	Shimadzu
6.	LC/MS/MS	8030	Shimadzu
7.	Operating Software	Lab Solution 5.72B	Shimadzu
8.	Micropipette (2-20 µL, 20-200 µL, 100-1000 µL, and 500-5000 µL)	-	Eppendorf
9.	Hot Air Oven	-	Associated scientific technologies
10.	Membrane Filter	0.22µ	Millipore
11.	Digital Analytical Balance	AUX 220	Shimadzu
12.	Refrigerator (2-8^{o}C)	GL335/388	LG Electronics
13.	Deep Freezer (-20 ^{o}C)	RQFV-265	REMI Corporation
14.	pH Meter	pH Tutor	Eutech Instruments
15.	Ultra-sonicator	Ultrasonic Steri-cleaner	Cyber-lab
16.	Vortex Shaker	CM-101	REMI
17.	Water Purification System	Elix 10, Milli-Q Gradient	Millipore
18.	Incubator	-	Thermolab
19.	Magnetic Stirrer	-	Electro quip
20.	Vacuum Pump	-	Millipore
21.	Vacuum Oven	NV 8510	NOVA Instrument Pvt. Ltd.

- **Equipment Condition**

Table 4.7: Tuning Parameter of MS/MS for drug and internal standard and source dependent parameter

Parameters	**QUE**	**RUTIN**	**GA**
Ion Source		Electro Spray ionization	
Polarity		Positive	Negative
Parent Ion	303.1	611.3	169.1
Daughter Ion	153	303.1	125.1
Dwell Time(msec)	100	100	100
Collision Energy (CE)	-35	-20	15
DL Temperature		250 °C	
Nebulizing Gas Flow		3.0 L/min	
Heat Block Temperature		450 °C	
Drying Gas Flow		15.0 L/min	

- **Chromatographic conditions**

Table 4.8: Chromatographic Conditions

Parameters	**Used**
Column	Kintex PFP, (50mm x 4.6mm, 2.6 µ)
Mobile Phase	0.2% Formic acid in water: Methanol (10:90% v/v)
Flow rate	0.500 mL/min,
Column oven temperature	40±0.3°C
Autosampler temperature	15±3°C
Volume of injection	10.0 µL
Detector	Mass detector (MS/MS)
Run time	3.0 minutes
Rinsing Volume	500µL
Needle Stroke	52 mm
Rinsing Speed	35.0µL/second
Sampling Speed	15.0µL/second
Purge Time	1.00 minute
Rinse Dip Time	1 second
Rinse Mode	Before and after aspiration

4.20.8 Determination of Selected Polyphenolic Compounds in Fractions:

The developed LC-MS/MS method was applied to analyze gallic acid, rutin, and quercetin content in CMEAF. The corresponding concentration was determined using the standard curve's regression equation.

4.21 Isolation and Characterization of Bioactive Compound from ethyl acetate fraction of *Citrus medica*

4.21.1 Isolation of bioactive compound(s) by preparative TLC

Preparative TLC plates with a thickness of 1mm were prepared using the same stationary and mobile phases as 3.16.1, to isolate the bioactive compounds that exhibited the anticoagulant activity. Based on the results, the highest % peak area of developed HPTLC. These areas were marked with pencil and scraped from the plates, and the substance was eluted from the silica with methanol, centrifuged, and filtered. To obtain the pure form of compounds, the filtrate was Sonicator for 90 minutes. Eluted compounds were further purified using the above preparative chromatography method. Finally, the components were identified by LCMS, HPTLC, and FTIR.

4.21.2 Characterization of purified bioactive compound

4.21.2.1 HPTLC Fingerprinting

The isolated compounds were produced at a concentration of 1 mg/ml. A TLC plate was applied with 5 ul of ferulic acid and 10 µl of the suitably pre-diluted compound(s) solution. Toluene, ethyl acetate, and formic acid (07:03:0.5) were used to develop the plates. Using LINOMAT-V and CAMAG TLC Scanner-3, the plate was scanned at 255 and 366 nm. The Rf value of each isolated chemical that was separated, together with the standard ferulic acid on the plate was recorded.

4.21.2.2 FT-IR analysis

IR spectrums were recorded using Perkin Elmer FT-IR Two ULTRA spectrophotometers in the 400-4000 cm-1 range at a flow rate of 16 (cm- 1/min) with Dichloride methane as solvent.